

SUPPLEMENTARY ONLINE DATA

FLIP_L induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity

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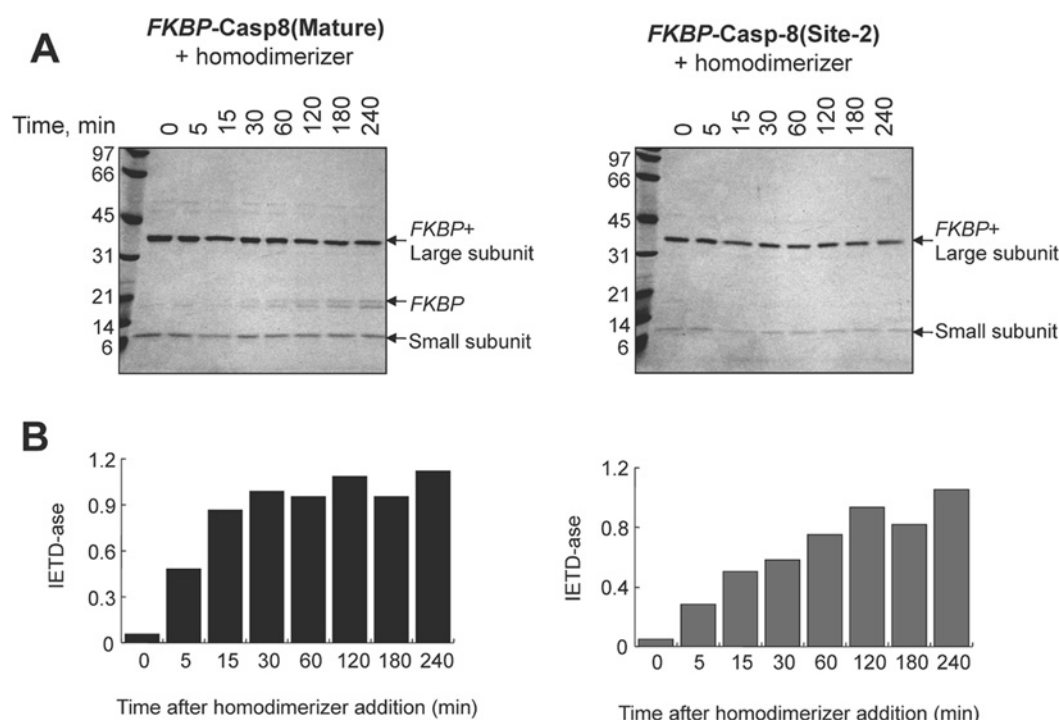


Figure S1 Stability of FKBP-caspase 8 activity

The majority of FKBP-caspase 8(mature) activation occurs within 15–30 min post-homodimerizer addition, prior to autocleavage of the pro-domain. **(A)** Kinetics of pro-domain autocleavage. Dimerized FKBP-caspase 8(mature) (left-hand panel) or control sample FKBP-caspase 8(Site-2 mutant) (right-hand panel) were dissolved in assay buffer at 0.5 μ M and incubated for the specified duration at 25°C. Reactions were stopped with loading buffer and analysed by SDS/PAGE (4–20% gels) stained with Coomassie Blue. The molecular mass in kDa is indicated on the left-hand side. **(B)** Kinetics of caspase activation by homodimerization. At the end of the incubation time, samples from **(A)** were diluted at 25 nM in caspase buffer and their IETD-ase activity [relative fluorescence units (RFU)/min] was immediately recorded. Most experiments for the present study were performed within 45 min–1 h post-dimerization.

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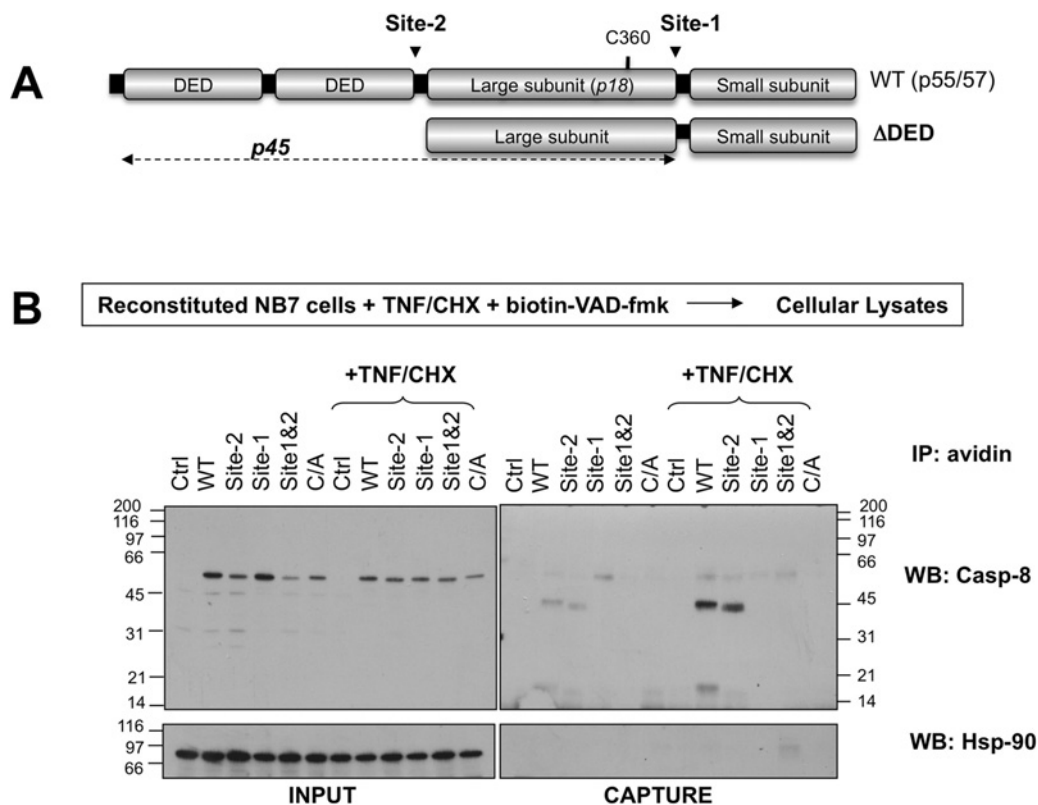


Figure S2 NB7 cells reconstituted with caspase 8 mutants

(A) Scheme of full-length caspase 8 and Δ DED-caspase 8 constructs used for cellular expression. (B) Active-site labelling of caspase 8 in cells. NB7 cells devoid of caspase 8 were transiently reconstituted with non-toxic amounts of caspase 8 mutants (50 ng/well for 12-well plates) and then treated with TNF α /CHX. B-VAD-fmk (50 μ M) was added to the cells 1 h prior to the addition of TNF α /CHX, followed by 18 h incubation. Cell lysates were subjected to pull-down using avidin beads followed by Western blotting against the specified proteins. The molecular mass in kDa is indicated on the left-hand side. IP, immunoprecipitation; WB, Western blot.

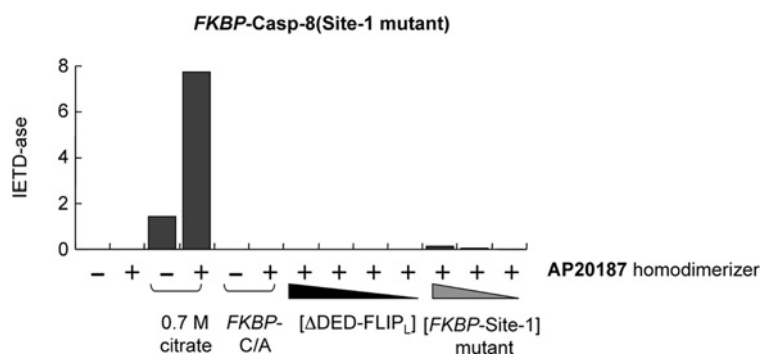


Figure S3 Activation of FKBP-caspase 8(Site-1 mutant) (50 nM) by the homodimerization compound (50 nM) in the presence of catalytically inactive FKBP-caspase 8(C/A) (500 nM), sodium citrate (0.7 M) or Δ DED-FLIP₁ (50–500 nM)

For experiments where the concentration of FKBP-caspase 8(Site-1) was varied, 50–500 nM enzyme was used with proportional amounts of dimerizer. IETD-ase is expressed as relative fluorescence units (RFU)/min.

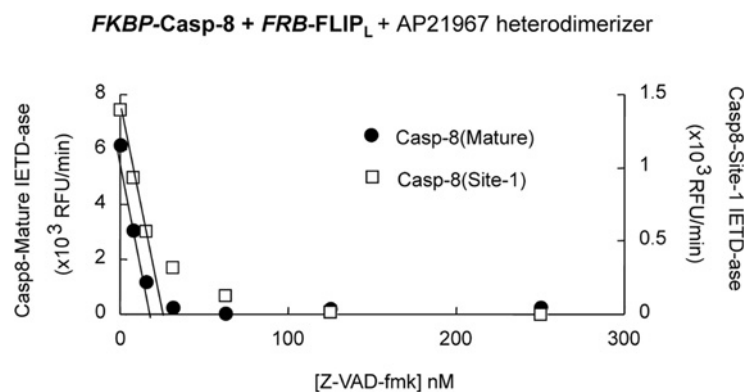


Figure S4 Active-site titration of caspase 8 heterodimers

FKBP-caspase 8 (50 nM based on absorbance at 280 nm) was activated with FRB-FLIP_L (250 nM) and heterodimerization compound (250 nM) as described in the Experimental section of the main text. Equal volumes of activated caspase 8 and Z-VAD-fmk solutions were mixed to generate 25 nM caspase 8 and the final concentrations of Z-VAD-fmk shown, followed by 30 min incubation at 25°C. The remaining enzymatic activity was quantified using the Ac-IETD-afc substrate at 30°C. The Figure shows that 80–100 % of the active sites are available for catalysis.

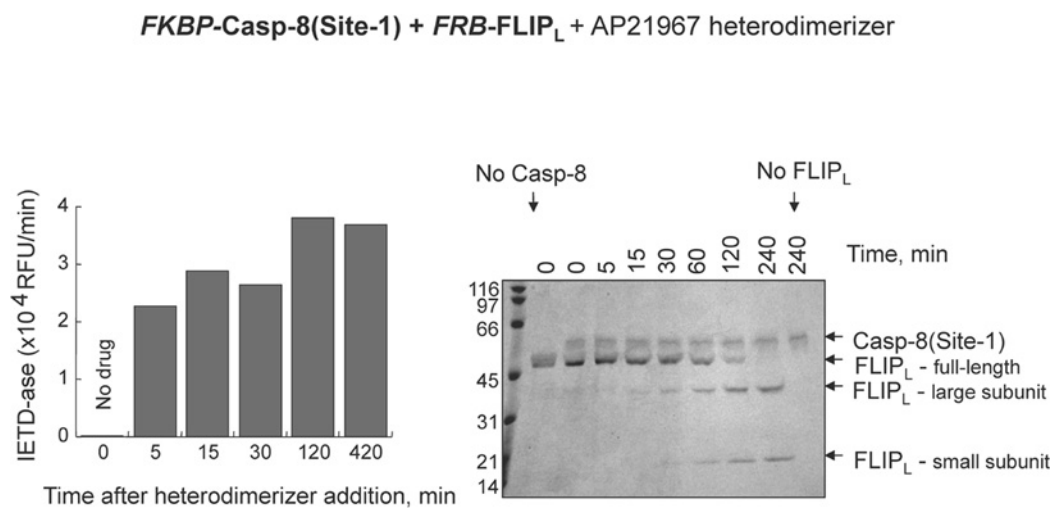


Figure S5 FLIP_L cleavage at LEVD/G by caspase 8 following its heterodimerization with FLIP

FKBP-caspase 8(Site-1 mutant) (500 nM), FRB-FLIP_L (2 μ M) and the heterodimerization compound (2 μ M) were mixed in the assay buffer and incubated at 25°C for the duration shown. The reaction was stopped with 3 \times SDS buffer and samples were run on SDS/PAGE (4–20% gels), followed by Coomassie Blue staining (right-hand panel). IETD-ase activity (left-hand panel) was determined for the samples containing FKBP-caspase 8(Site-1 mutant). The molecular mass in kDa is indicated on the left-hand side.

Table S1 Catalytic parameters of FKBP-caspase 8 homodimers at 30°C(a) k_{cat} (s^{-1})

Caspase 8 dimer	Mature	Site-2 mutant
IETD	0.325 ± 0.004	0.45 ± 0.004
LEHD	1.08 ± 0.05	1.56 ± 0.1
DEVD	0.12 ± 0.003	0.17 ± 0.003
LEVD	0.023 ± 0.0005	0.033 ± 0.0005

(b) K_m (μM)

Caspase 8 dimer	Mature	Site-2 mutant
IETD	21.7 ± 1.1	28.3 ± 1.1
LEHD	135 ± 13.24	160 ± 22
DEVD	8 ± 2.5	24.9 ± 1.7
LEVD	0.9 ± 0.2	2.5 ± 0.2

(c) k_{cat}/K_m ($\text{M}^{-1}\cdot\text{s}^{-1}$)

Caspase 8 dimer	Mature	Site-2 mutant
IETD	1.5×10^4	1.6×10^4
LEHD	0.8×10^4	0.97×10^4
DEVD	0.5×10^4	0.7×10^4
LEVD	1.2×10^4	1.3×10^4

Table S2 Catalytic parameters of FKBP-caspase 8–FRB–FLIP_L heterodimers at 30°C(a) k_{cat} (s^{-1})

Caspase 8 dimer	Mature–FLIP _L	Mature–FLIP _L (D/A)	Site-2–FLIP _L	Site-2–FLIP _L (D/A)	Site-1–FLIP _L	Site-1–FLIP _L (D/A)	Site-1+2–FLIP _L	Site-1+2–FLIP _L (D/A)
IETD	0.27 ± 0.006	0.36 ± 0.003	0.53 ± 0.01	0.52 ± 0.01	0.073 ± 0.002	0.121 ± 0.016	0.09 ± 0.004	0.124 ± 0.003
LEHD	0.89 ± 0.02	1.15 ± 0.02	1.41 ± 0.02	1.48 ± 0.01	0.18 ± 0.002	0.196 ± 0.02	0.2 ± 0.001	0.215 ± 0.004
DEVD	0.10 ± 0.005	0.129 ± 0.003	0.18 ± 0.008	0.18 ± 0.005	0.05 ± 0.003	0.066 ± 0.002	0.06 ± 0.003	0.071 ± 0.002
LEVD	0.02 ± 0.0006	0.03 ± 0.0006	0.049 ± 0.001	0.03 ± 0.0006	0.02 ± 0.001	0.028 ± 0.0007	0.029 ± 0.001	0.032 ± 0.006

(b) K_m (μM)

Caspase 8 dimer	Mature–FLIP _L	Mature–FLIP _L (D/A)	Site-2–FLIP _L	Site-2–FLIP _L (D/A)	Site-1–FLIP _L	Site-1–FLIP _L (D/A)	Site-1+2–FLIP _L	Site-1+2–FLIP _L (D/A)
IETD	16.5 ± 1.5	20.4 ± 0.7	26.5 ± 1.9	25.7 ± 2	31.5 ± 3.4	59.4 ± 2.1	27.6 ± 4.6	59.3 ± 3
LEHD	60.1 ± 4.1	73.5 ± 3.7	64.5 ± 3.2	71.9 ± 2.1	123.4 ± 30.3	122.7 ± 3.6	57.8 ± 9.4	112.5 ± 5
DEVD	21.8 ± 3.8	20.3 ± 2.1	25.5 ± 3.9	21.9 ± 2.4	101.9 ± 17	172.2 ± 11	75.7 ± 10	175.7 ± 11
LEVD	4.0 ± 0.46	3.7 ± 0.4	5.2 ± 0.9	4.1 ± 0.2	29.2 ± 5	40.9 ± 3	21.6 ± 3	42.6 ± 2.7

(c) k_{cat}/K_m ($\text{M}^{-1}\cdot\text{s}^{-1}$)

Caspase 8 dimer	Mature–FLIP _L	Mature–FLIP _L (D/A)	Site-2–FLIP _L	Site-2–FLIP _L (D/A)	Site-1–FLIP _L	Site-1–FLIP _L (D/A)	Site-1+2–FLIP _L	Site-1+2–FLIP _L (D/A)
IETD	1.66×10^4	1.76×10^4	2.01×10^4	2.05×10^4	0.23×10^4	0.204×10^4	0.35×10^4	0.209×10^4
LEHD	1.49×10^4	1.56×10^4	2.18×10^4	2.06×10^4	0.14×10^4	0.159×10^4	0.35×10^4	0.191×10^4
DEVD	0.48×10^4	0.63×10^4	0.71×10^4	0.82×10^4	0.04×10^4	0.038×10^4	0.08×10^4	0.040×10^4
LEVD	0.72×10^4	0.93×10^4	0.72×10^4	1.11×10^4	0.07×10^4	0.070×10^4	0.13×10^4	0.073×10^4

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